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Sensitive Detection and Quantification of Particle-Associated Reverse Transcriptase in Plasma of HIV-1-Infected Individuals by the Product-Enhanced Reverse Transcriptase (PERT) Assay

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Tests for the enzyme reverse transcriptase (RT) should permit the detection of all infectious retroviruses, provided that these are present as extracellular particles. The capability of a new procedure, named product-enhanced reverse transcriptase (PERT) assay, to detect HIV-1 in fresh human plasma was compared with that of the polymerase chain reaction (PCR) for viral RNA. Both procedures had identical dilution endpoints corresponding to 10^2 particles/ml. All 30 samples from HIV-1 positive patients at different stages contained RT activity whose level was significantly correlated with viral RNA and corresponded to 553–417,000 particles/ml. In HIV-1 low titer performance and seroconversion panels, the PERT assay detected more positives than PCR for viral RNA. Three of 160 blood donors exhibited elevated RT activity, indicating a prevalence of 1.9% (95% CI 0.4–5.3%). One positive donor, with laboratory parameters suggesting a mild chronic liver impairment, exhibited RT activity comparable to that of HIV positives, but was consistently negative by various tests for hepatitis viruses, cytomegalovirus, the HIVs and HTLVs. The results suggest that the PERT assay is more sensitive for detection of HIV-1 contamination of plasma than RNA PCR. However, it is not affected adversely by viral sequence variability, and may therefore, also detect HIV-1 subtype O, and additional retroviruses as yet undetectable by PCR.

KEY WORDS: reverse transcriptase, RNA-dependent DNA polymerase

INTRODUCTION

Tests for the human immunodeficiency viruses HIV-1 and HIV-2 have been improved continuously over the past decade. In addition to serological tests, molecular methods such as the polymerase chain reaction (PCR) permit the reliable identification of HIV-infected individuals or materials. Recent experience with HIV-1 subtype O infections has shown however, that sequence variability may impair seriously the sensitivity of these assays [Nkengasong et al., 1993; Loussert-Ajaka et al., 1994; Dondero et al., 1994]. A test for retroviruses that does not depend on viral nucleic acid sequence and is not impaired by sequence variation would thus be of considerable interest.

All replication-competent retrovirus particles contain the enzyme reverse transcriptase (RT). Sufficiently sensitive methods for RT should thus allow detection of not only all variants of HIV, but all infectious retroviruses. Recently, we described an ultrasensitive RT test [Pyra et al., 1994] and similar procedures have also been developed by others [Silver et al., 1993; Heneine et al., 1995]. The assay, which we named product-enhanced reverse transcriptase (PERT) assay, is 10^6 to 10^7 times more sensitive than a conventional RT test and detects as little as 10^{-9} units of recombinant murine leukemia virus (MuLV) RT, which corresponds to 2.1×10^2 molecules, or the enzyme present in 3 to 11 virus particles. Preliminary experiments indicated that the test detects a wide variety of retroviruses, suggesting that it might be used as a universal retrovirus test.

The present study was undertaken to assess the assay's ability to detect and quantitate particle-associated RT in plasma of HIV-1-infected patients and to estimate the frequency of such activity in healthy blood donors.

MATERIALS AND METHODS

Sample Preparation

Ten ml EDTA blood was taken from HIV-1-infected patients seen at the Division of Infectious Diseases of the

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Zurich University Hospital's Department of Medicine, after informed consent was obtained, or from unselected blood donors of the Zurich Red Cross Transfusion Center. The anonymized samples were sent to the laboratory, where the cells were removed by centrifugation within 12 hours after collection. Serum or plasma aliquots of 0.25 ml of the HIV-1 low titer performance panel PRB 104 and seroconversion panels E, N, P, Q, W, X, and Y were generously provided by Boston Biomedica Inc. (BBI), West Bridgewater, Massachusetts. Sample pretreatment and virus concentration by ultracentrifugation were as published previously [Pyra et al., 1994]. Three μl of resuspended pellet, representing 100 μl plasma (25 μl for the BBI panels), were tested by PERT assay or RNA PCR.

PERT Assay

The published procedure was used [Pyra et al., 1994]. Briefly, genomic RNA from phage MS2 was used as template for reverse transcription together with the synthetic oligonucleotide primer RT-1. After addition of the primer template to the RT reaction mixture, which contained all elements required for RT-mediated cDNA synthesis, including Mg^{2+} as the divalent cation, 3 μl of a resuspended plasma pellet was added and reverse transcription was allowed to proceed at 37°C for 5 hours. The MS2 cDNA thus synthesized was amplified selectively by 25 cycles of PCR using primers RT-1 and RT-2. The amplification product was analyzed by ELISA as described previously [Pyra et al., 1994], unless noted otherwise. All testing was done blinded and in duplicate. For standardization, culture supernatant of the HIV-1 producer cell line H9/HTLV-III_B was diluted serially in human plasma, aliquoted and stored frozen at −80°C. An aliquot of each dilution was included subsequently in each run of sample preparation and PERT assay. Serological test panels were examined with some minor modifications. Reverse transcription was allowed to proceed only for 90 min and amplification by PCR was carried out in a reduced volume of 55 μl in a heat-resistant microtiter plate. In addition, virus-containing plasma was replaced by serial dilutions of recombinant murine leukemia virus (MuLV) RT (Boehringer Mannheim (Schwarz) AG, Rotkieuz) as an activity standard.

RNA PCR

The reaction conditions for cDNA synthesis with sequence-specific primers and for amplification by PCR were as described previously [Pyra et al., 1994], except that different primers were used and the number of cycles was adjusted to the type of analysis. For quantitation of HIV-1 RNA by endpoint dilution, primer HIV-G1 was used for cDNA synthesis of the *gag* region, and DNA was amplified for 40 cycles with primers HIV-G1 and HIV-G2 [Böni and Schüpbach, 1993a]. For quantitation of HIV-1 RNA by comparison with external RNA standards, cDNA synthesis of the long terminal repeat (LTR)-region was carried out with primer HIV-L3, and DNA was amplified for 30 cycles with primers HIV-L1 and HIV-L3 [Böni and Schüpbach, 1993a]. For diagnostic RNA PCR, primers HIV-L3 and HIV-G2 or HIV-G9 [Böni and Schüpbach, 1993b] were used for cDNA synthesis. Amplifications were carried out for 35 cycles with primers HIV-L1 and HIV-L3, HIV-G1 and HIV-G2, or SK145 [Krone et al., 1990] and HIV-G9.

In order to obtain a synthetic target sequence of the *gag* region for RNA PCR, plasmid pGHIV-H2 was generated by subcloning the 0.63 kb Hind III fragment of plasmid pBT-1, which contains the 8.9 kb Sst I fragment of the HIV-1 LAI isolate, into the Hind III site of plasmid pGem3. After linearization of plasmid pGHIV-H2 with Eco RI, positive strand RNA was synthesized using the Riboprobe System and RNA polymerase SP6 (Promega Corporation, Madison, WI).

Detection of Amplified DNA Products by Southern Blot

Ten μl of each amplified DNA were separated on an agarose gel and blotted subsequently onto a nylon membrane (Hybond-N plus, Amersham International, Amersham, UK), according to the manufacturer's instructions for alkaline transfer. Oligonucleotide probes RT-3 [Pyra et al., 1994] or HIV-L2 [Böni and Schüpbach, 1993a], labeled at the 5′-end with ^{32}P, were used for hybridization of MS2- or, respectively, HIV-1-derived DNA following standard protocols [Sambrook et al., 1989]. To visualize the radioactivity, membranes were exposed to Fuji RX X-ray films and for quantitation, bands were cut out and counted in a liquid scintillation counter.

Detection of Amplified DNA Products by ELISA

Amplified DNA products were analyzed with a nonisotopic detection system which is based on solution hybridization of the amplified DNA with two oligonucleotide probes that are labeled with biotin and digoxigenin, respectively. The hybrid molecules were then bound to a solid phase and detected by enzyme immunoassay [Böni and Schüpbach, 1993b]. For the detection of HIV-1-derived DNA, the reaction conditions were as published [Böni and Schüpbach, 1993b], except that additional oligonucleotide probes were used. Hybridization was with probes HIV-L2-Bio (5′-biotin-HIV-L2) [Böni and Schüpbach, 1993a] and HIV-L7-DIG (5′-digoxigenin-d[GCAC-TCAAGGCA-AGCTTTATTGAGGC]-3′) for DNA amplified with primers HIV-L1 and HIV-L3, with probes HIV-G3-Bio (5′-biotin-HIV-G3) [Böni and Schüpbach, 1993a] and HIV-G7-DIG (5′-digoxigenin-d[AATAGTAAGAAT-GTAT-AGCCCT]-3′) for DNA amplified with primers HIV-G1 and HIV-G2, and with probes SK102-Bio and HIV-G13-DIG for DNA amplified with primers SK145 and HIV-G9 [Böni and Schüpbach, 1993b]. Detection of amplified MS2 DNA of the PERT assay was as published [Pyra et al., 1994].

Serological Analyses

Data from serological analyses of the low titer performance and seroconversion panels were collected from various sources. Results of the screening assays were compiled from the data sheets provided by the distributor of the panels and from performance data obtained

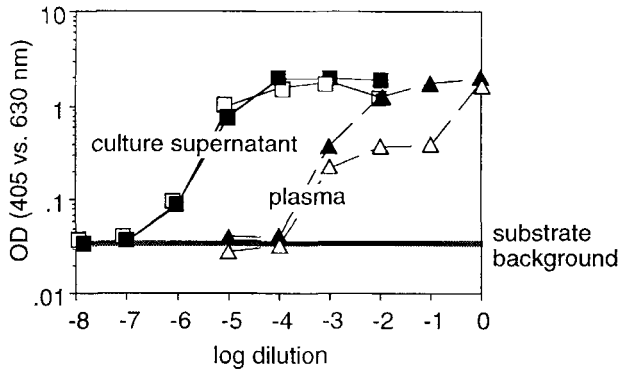

Fig. 1. Comparative sensitivity of PERT assay and RNA PCR. A supernatant from an HIV-1 producer cell line and the plasma sample of an HIV-1-infected individual were serially diluted and equal amounts of each dilution were tested in parallel by PERT assay and by RNA PCR of the viral *gag* region. Closed symbols, PERT assay; open symbols, RNA PCR.

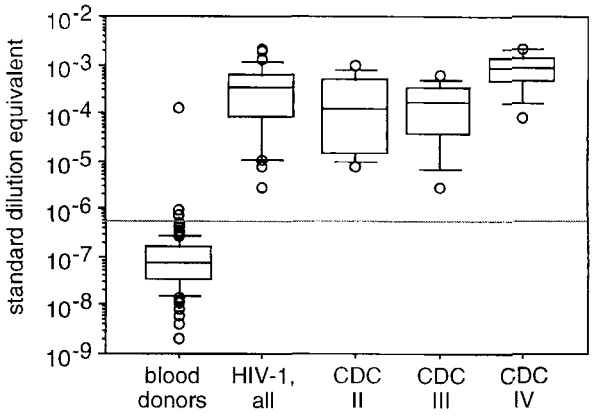

Fig. 2. Particle-associated RT activity in plasma of blood donors and HIV-1-positives. RT activity is expressed as the dilution equivalent of the HIV-1 containing standard. The horizontal lines in the box plot presentation indicate the 10th, 25th, 50th, and 90th percentiles. The horizontal line across the figure represents the cut-off.

from kit manufacturers on a confidential basis [Schüpbach, 1996]. Only data from kits licenced in the U.S. or Europe, including the most sensitive tests currently available on the European market, were considered. Western blot results were based exclusively on FDA licenced Western blot assays and were taken from the data provided by the distributor of the panels. The interpretation of Western blot results followed the ASTPHLD/CDC recommendations [Association of State and Territorial Public Health Laboratory Directors and AIDS Program, 1989]. HIV p24 antigen data were taken from the data sheets of the panels. They are based on the Abbott HIV antigen assay using procedures not involving immune complex dissociation.

RESULTS

In order to establish the detection limit of the procedure for HIV-1 particles, filtered supernatants from an HIV-1 producer cell line and plasma from an HIV-1 infected individual were diluted serially and equal amounts of the ultracentrifugation pellets were tested for RT activity by the PERT Assay and for HIV-1 RNA by PCR. Figure 1 shows that the dilution endpoints of the two procedures were identical. RNA PCR of a standard series containing 10^7 to 10^0 copies of in vitro HIV-1*gag* RNA indicated a detection limit of about 10^1 copies for the RNA PCR (not shown). Thus, the detection limit of the PERT assay in this experiment was in the order of five HIV-1 particles, which is in agreement with earlier estimates [Pyra et al., 1994]. Furthermore, dilution series with recombinant HIV-1 RT indicated a detection limit of less than 50 RT molecules (not shown).

In order to evaluate the ability of the PERT assay to distinguish HIV-1 infected individuals from uninfected controls, plasma samples from 30 HIV-1 patients and 160 unselected blood donors were tested (Fig. 2). The median RT activity of HIV-1 positives was 3.6 orders of magnitude higher than that of the blood donors. The two groups did not overlap, with the single exception of an HIV-negative blood donor whose plasma exhibited a level of RT activity that was comparable to those of HIV-positives. This donor was negative by serological tests for HBsAg, HCV, CMV, and HIV-1/2 that had been carried out repeatedly over a period of 28 months at the blood donation center. The only abnormal finding present consistently during this interval was a slightly elevated level of alanine aminotransferase. Further examination of this RT-positive blood sample by PCR yielded negative results for DNA and RNA of HIV-1 and HIV-2, for RNA of HTLV-1 and HTLV-2, for DNA of HBV, and for RNA of HCV.

Based on the RT values of the blood donors and after the obvious outrunner had been excluded, a cut-off of positivity was defined as the mean activity (0.117×10^{-6}) plus 3 standard deviations (0.133×10^{-6}), which was equal to 0.52×10^{-6}. Based on this cut-off value, slightly elevated levels of RT activity (1.4 and 1.8, respectively) were present in two additional donors. Accordingly, the prevalence of elevated filterable particle-associated RT activity in fresh plasma of blood donors was 3/160 (1.9%; 95% CI = 0.4–5.3%). The observed diagnostic sensitivity of the PERT assay in the 30 HIV-1 positives was 100% (95% CI 88.4–100%). Regarding the levels of RT activity, CDC 87 stages II and III showed a similar distribution with a median of 235 × cut-off, while stage IV samples ranged significantly higher with a median of 1580 × cut-off (t-test; $P < .001$).

Particle concentrations based on RT activity were then compared with those derived from RNA PCR of the LTR region (Fig. 3). Quantification by both procedures was again based on the supernatant standards. Separate experiments indicated that a 10^{-6} dilution of this standard contained 10^2 particles/ml (not shown). The viral loads determined by the two methods (Fig. 3C) showed a good correlation ($R^2 = 0.714$; $P = .0005$, t-test). Similar results for RNA PCR were obtained with two additional primer pairs and probes from the *gag* region (not shown). Remarkably, the concentrations de-

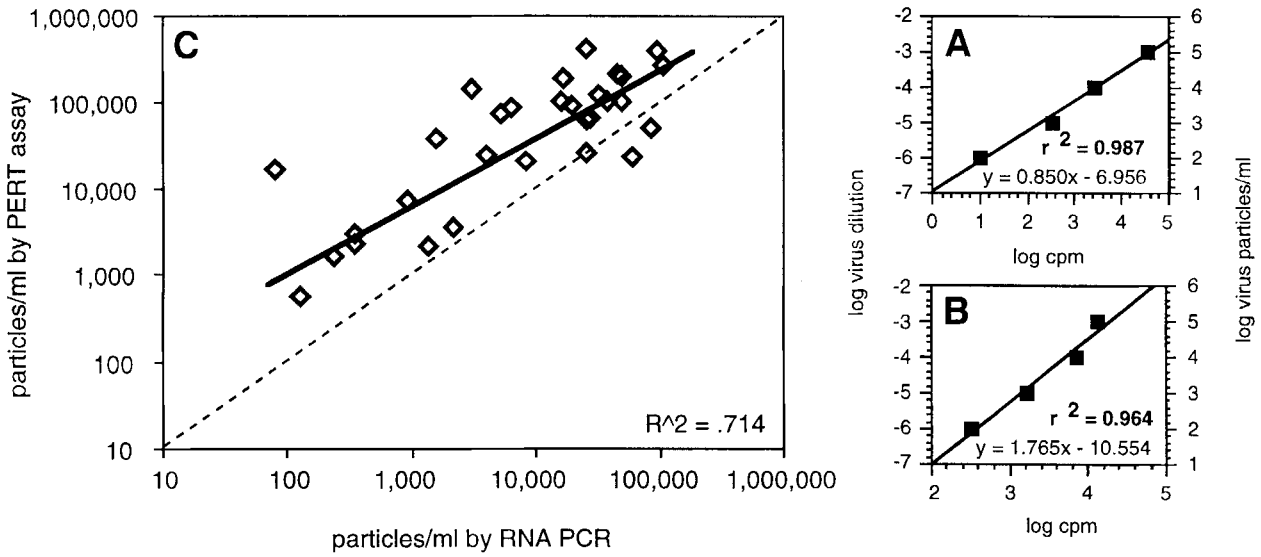

Fig. 3. Quantification of HIV-1 in plasma by RNA PCR of the viral LTR region and by PERT assay. The viral load in plasma from 30 HIV-1-infected patients was determined by the two methods, using Southern blot analysis and external HIV-1 standards as quantitative references. **A:** External standard curve used for the quantification of HIV-1 by RNA PCR. **B:** External standard curve for the quantification of RT activity by PERT assay (same standard as in A). **C:** Viral load estimate in particles/ml plasma of the 30 HIV-1-infected patients.

rived from the RT activity were significantly higher than those based on RNA PCR (P = .0005, t-test). RNA PCR indicated concentrations from 80 to 110,000 particles/ml (median 16,600), while the PERT assay yielded 553–417,000 particles/ml (median 64,300). On the average, the viral load by PERT assay was about four times higher than by RNA PCR. One sample in particular indicated a concentration of only 80 particles/ml by RNA PCR, but more than 16,400 particles/ml when tested for RT activity.

In order to assess further the sensitivity of the PERT assay, an HIV-1 low titer performance panel and 7 seroconversion panels were tested for particle-associated RT activity and for viral RNA. Since the HIV-1 supernatant standards had been used up, serial dilutions of recombinant MuLV RT were now used as activity standards. The cut-off of the PERT assay was again set at the mean plus 3 standard deviations of a control population of 107 individuals different from the blood donors shown in Figure 2. This cut-off corresponded to 10 nU/ml. Figure 4 shows the RT activity of these panels in comparison with the RNA PCR results and the serological data of antibody screening, Western blot and antigen p24. Both RNA PCR and PERT assays yielded similar results, although the positivity rate of the PERT assay was somewhat higher (66% vs. 58%, n.s.). In the low titer performance panel, 8 antigen-negative samples were positive by PERT assay, 7 of these were also positive by RNA PCR. The sample negative by RNA PCR was low positive (A/CO = 2.56) in the PERT assay. In the 7 seroconversion panels, the first positive results by RNA PCR or PERT assay usually coincided with the first positive antigen result, with the exception of panels N and W. No antigen was detectable in panel N; RT just about the cut-off level demonstrated a very low virus load just below the detection limit of RNA PCR. Since all samples of this panel are antibody-positive, panel N may in fact represent a postseroconversion rather than a seroconversion panel. In panel W, RT activity was close to cut-off at day 14, just above cut-off at day 28, completely negative at day 30, but strongly positive at day 35 when RNA PCR also became positive. Antigen became positive two days later, demonstrating the rapid increase of the viral load in the preseroconversion period. After the appearance of antibodies, antigen usually became rapidly undetectable, while PERT assay and RNA PCR remained positive. In panels E, Q, X, and Y, the RT activity was reduced significantly, however, reflecting the rapid reduction of the viral load observed in the postseroconversion period. No reduction was observed in panel W and the RT load remained high four months after the first detection of the virus.

DISCUSSION

The above results suggest that a new assay for retroviral reverse transcriptase activity detects HIV-1 particles with greater sensitivity than RNA PCR. This is demonstrated by dilution endpoints (Fig. 1), the testing of plasma from patients at different stages of infection (Figs. 2, 3), and of serum and plasma samples of low titer performance and seroconversion panels (Fig. 4). The test detects less than 100 HIV-1 particles/ml and the specificity with plasma separated from cells within 12 h after the blood was drawn is good (Fig. 2). The PERT assay thus compares well with the most sensitive sequence-specific methods described by others [Piatak et al., 1993; Bruisten et al., 1993; Ho et al., 1995].

In contrast to sequence-specific procedures, whose sensitivity may be hampered by genomic variability, as is the case for HIV-1 subtype O infection [De Leys et al., 1990; Gürtler et al., 1994; van den Haesevelde et al., 1994], the PERT Assay is not affected adversely

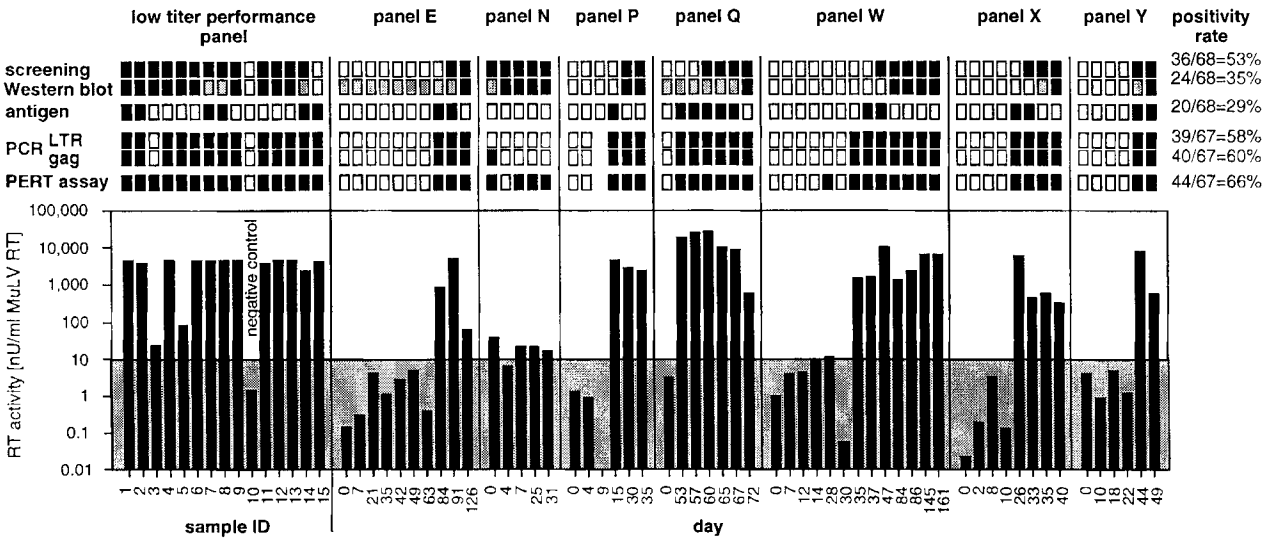

Fig. 4. Sensitivity of different tests in an HIV-1 low titer performance and 7 seroconversion panels. Results of serological assays were compiled from the sources described in Materials and Methods. Screening positivity represents positive results with at least two different kits, whereas for Western blots for each sample, the result of the most sensitive assay is recorded. RNA PCR with primers and probes from the LTR and *gag* regions of the viral genome and PERT assay were performed in our laboratory. Results of the PERT assay are represented both qualitatively (boxes) and quantitatively (column diagrams). Results of the other tests are shown only qualitatively. Black box, positive; empty, negative; shaded, indeterminate.

by sequence variation. Thus, all conceivable variants of HIV-1 can be detected. The significantly higher virus loads obtained with the PERT assay in Figure 3 suggest that sequence variation may sometimes lead to considerable underestimation of the HIV load by PCR and other sequence-based procedures. The PERT assay may also give a better approximation of the virus fraction that is biologically active, i.e., viral RNA detection may remain unaffected even after the virus has been rendered noninfectious by physical or chemical inactivation. It may also be expected that when assessing the effect exerted by proteinase inhibitors on RT, a test for RT activity would be more suitable than a test for viral RNA.

Obviously, a positive PERT assay result yields no clue regarding its origin. Thus, the cause of activity in the three positive blood donors remains unknown. One of these donors had a repeatedly elevated liver enzyme compatible with a mild form of chronic hepatitis, while clinical information was not available on the two low-positive donors. Possible causes for the RT activity in these cases include genuine RTs from exogenous or endogenous retroviruses, or enzymes from other infectious agents or from cells that may have a low capability of synthesizing a cDNA from heteropolymeric RNA templates. It is well known that certain bacterial DNA polymerases, such as DNA polymerase I from *E. coli,* are capable of reverse transcription. However, the involvement of bacterial polymerases in these cases is unlikely, since the samples were filtered carefully before testing. Release of cellular polymerases before the removal of blood cells cannot be excluded. We and others [Silver et al., 1993] found that lysates of normal unstimulated peripheral blood mononuclear cells (PBMC) and extracts from some tissue culture cells are positive by this type of test (not shown). However, in another borderline positive blood donor we found an RT activity that could be transmitted in vitro to a series of fresh PBMC (unpublished). Thus, the RT activity found in the three donors is not necessarily nonspecific and needs further investigation. Consequently, it is not possible at present to give a precise figure for the diagnostic specificity of the PERT assay.

In contrast to PCR or other sequence-specific nucleic acid detection methods, which can only be used for viruses whose genomic sequence is known exactly, the PERT assay does not rely on viral sequence information. Consequently, even hitherto unknown retroviruses can be detected, provided that they present as particles with an enzymatically active RT, a property which is, however, indispensable for infectivity. An involvement of retroviruses has been postulated for several other, particularly autoimmune and neoplastic, disorders. The PERT assay will be important in verifying or dismissing such claims.

The PERT assay may also become valuable for safety testing of cell-free biological products for use in humans, e.g., fresh plasma, blood coagulation factors, or vaccines. The risk of inadvertent transmission of infectious retroviruses through such products, or allo- and xenotransplants, could certainly be reduced further [Nowak, 1995]. After some additional development aimed at simplifying the sample pretreatment step, the test may also be suitable for the screening of blood donors.

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REFERENCES

Association of State and Territorial Public Health Laboratory Directors and AIDS Program (1989): Interpretation and use of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infections. Morbidity and Mortality Weekly Reviews 38 (No. S-7):1–7.

Böni J, Schüpbach J (1993a): Primer extension analysis provides a sensitive tool for the identification of PCR-amplified DNA from HIV-1. Journal of Virological Methods 42:309–322.

Böni J, Schüpbach J (1993b): Sensitive and quantitative detection of PCR-amplified HIV-1 DNA products by an enzyme linked immunoassay following solution hybridization with two differently labeled oligonucleotide probes. Molecular and Cellular Probes 7:361–371.

Bruisten S, van Gemen B, Koppelman M, Rasch M, van Strijp D, Schukkink R, Beyer R, Weigel H, Lens P, Husman H (1993): Detection of HIV-1 distribution in different blood fractions by two nuclei acid amplification assays. AIDS Research and Human Retroviruses 9:259–265.

De Leys R, Vanderborght B, van den Haesevelde M, Heyndrickx L, van Geel A, Wauters C, Bernaerts R, Saman E, Nijs P, Willems B, Taelman H, van der Groen G, Piot P, Tersmette T, Huisman JG, Van Heuverswijn H (1990): Isolation and partial characterization of an unusual human immunodeficiency retrovirus from two persons of west-central African origin. Journal of Virology 64: 1207–1216.

Dondero TJ, Hu DJ, George JR (1994): HIV-1 variants: Yet another challenge to public health. Lancet 343:1376.

Gürtler LG, Hauser PH, Eberle J, von Brunn A, Knapp S, Zekeng L, Tsague JM, Kaptue L (1994): A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. Journal of Virology 68:1581–1585.

Heneine W, Yamamoto S, Switzer WM, Spira TJ, Folks TM (1995): Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. The Journal of Infectious Diseases 171:1210–1216.

Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M (1995): Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123–126.

Krone WJA, Sninsky JJ, Goudsmit J (1990): Detection and characterization of HIV-1 by polymerase chain reaction. Journal of Acquired Immune Deficiency Syndrome 3:517–524.

Loussert-Ajaka I, Ly TD, Chaix ML, Ingrand D, Saragosti S, Couroucé AM, Brun-Vézinet F, Simon F (1994): HIV-1/HIV-2 seronegativity in HIV-1 subtype O infected patients. Lancet 343:1393–1394.

Nkengasong JN, Peeters M, van den Haesevelde M, Musi SS, Willems B, Ndumbe PM, Delaporte E, Perret J-L, Piot P, van den Groen G (1993): Antigenic evidence of the presence of the aberrant HIV-1 ANT70 virus in Cameroon and Gabon. AIDS 7:1536–1537.

Nowak R (1995): FDA puts the brakes on xenotransplants. Science 268:630–631.

Piatak M, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk K-C, Hahn BH, Shaw GM, Lifson JD (1993): High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 259:1749–1754.

Pyra H, Böni J, and Schüpbach J (1994): Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proceedings of the National Academy of Sciences USA 91:1544–1548.

Sambrook J, Fritsch E, and Maniatis T (1989): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Schüpbach J (1996): Licensing of diagnostic HIV Kits—to keep a sensible standpoint in an area of permanent revolution. Clinical and Diagnostic Virology, in press.

Silver J, Maudru T, Fujita K, Repaske R (1993): An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions. Nucleic Acids Research 21:3593–3594.

van den Haesevelde M, Decourt J-L, De Leys RJ, Vanderborght B, van der Groen G, van Heuverswijn H, Saman E (1994): Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. Journal of Virology 68:1586–1596.